Responses of grapevine cells to physiological doses of ethanol, among which induced resistance to heat stress

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Keywords: cell cultures, ethanol, hormesis, RNA-seq, small heat shock proteins, Vitis vinifera.

Abbreviations :

DEA – Differential Expression Analysis

DEGs – Differentially Expressed Genes

EtOH – Ethanol

GE – Gene Enrichment

GO – Gene Ontology

HS – Heat Shock

HSP – Heat-Shock Proteins

OD – Optical Density

PCA – Principal Component Analysis

RNA-seq - RNA sequencing

sHSP – small Heat-Shock Proteins

TPI - Total Polyphenol Index

Abstract

Grapevine naturally endures stresses like heat, drought, and hypoxia. A recent study showed very low oxygen levels inside grape berries, linked to ethanol content. Other studies have established the link between ethanol and tolerance to various stresses: heat stress, drought, and high salinity. The causes of such a tolerance are not well understood. In our study, three-week-old Gamay calli, *Vitis vinifera*, were characterised for their endogenous oxygen levels and endogenous ethanol concentration. Subsequently, a transcriptomic study of these cells was conducted, 6 and 24 hours after treatment with 1 mM ethanol. After 6 hours, ethanol addition led to 386 differentially expressed genes, with a notable upregulation of genes related to heat response, especially small Heat Shock Proteins (sHSPs). Further experiments showed that ethanol priming in grape cells or in *Arabidopsis* seedlings reduced pigment and electrolyte leakage under heat stress, respectively. This study supports the idea that ethanol priming helps protect plants against heat stress and provides a valuable RNA-seq dataset for further research into the underlying mechanisms, sHSPs playing a potentially crucial role in this adaptive response.

Introduction

Hypoxia occurs in plant cells due to the insufficient availability of oxygen (Loreti and Perata, 2020). Two types of hypoxia can be distinguished: environmental hypoxia can be caused by specific environmental cues, while developmental hypoxia occurs in tissues and organs in well-oxygenated surroundings, under normal atmosphere oxygen availability (Jethva *et al*., 2022).

Environmental hypoxia is a condition periodically encountered by terrestrial plants, often in waterlogged soils (Daniel and Hartman, 2024). This phenomenon can also be induced by rising temperatures, as dissolved oxygen levels decrease with higher temperatures (Chapra *et al*., 2021) and increased microbial activity further depletes oxygen (Alkorta *et al*., 2017). Additionally, hypoxia occurs at high altitudes due to the reduction in oxygen partial pressure (Abbas *et al*., 2022).

Developmental hypoxia naturally occurs in bulky or dense tissues like seeds, fruits, teguments, and roots where oxygen diffusion is limited (Brazel and Graciet, 2023). It also occurs during rapid cell proliferation when the respiratory rate is intrinsically high: oxygen is consumed faster than it diffuses into the plant tissue (Geigenberger, 2003). For instance, Xiao *et al* (2018) reported very low levels of oxygen in grape berries during growth and late-ripening processes. Furthermore, developmental hypoxia has been observed in growing potato tubers (Geigenberger *et al*., 2000), in the embryos of faba bean (*Vicia faba*) and green pea (*Pisum sativum*) (Rolletschek *et al*., 2002), and in seeds during the germination process (Borisjuk and Rolletschek, 2009). Oxygen levels have been observed to decrease to hypoxic levels during rapid cell proliferation in specific organs, such as the shoot apical

69 meristem and developing monocot anthers in *Zea mays* (Dukowic-Schulze and van der Linde, 2021),
70 and during grapevine bud burst (Meitha *et al*., 2018).

71 Low oxygen concentrations induce cellular stress due to decreased ATP production, depletion of
72 energy reserves, and accumulation of metabolic end-products notably ethanol (Jethva *et al*., 2022)
73 through fermentative metabolism (Raymond *et al*., 1995). Ethanol also accumulates during particular
74 plant developmental stages listed above: embryo and seedling development, fruit development, and
75 maturation (Diot *et al*., 2024). It also accumulates in response to many other stresses including air
76 pollution (sulphur dioxide and ozone exposure), water deficit, and freezing (Kimmerer and Kozlowski,
77 1982).

78 The impact of ethanol on plant physiology, particularly at low doses, is an emerging area of
79 research as it could be a potential molecular marker of hypoxia (Diot *et al*., 2024). While high
80 concentrations of ethanol are known to be toxic to plants, the few studies having investigated the
81 effects of low physiological doses of ethanol on plants show beneficial outcomes, like resistance to
82 heat stress (Matsui *et al*., 2022; Todaka *et al*., 2024), resistance to drought (Bashir *et al*., 2022) or
83 resistance to high salinity (Das *et al*., 2022).

84 Matsui *et al*. (2022) have shown that exposure to physiological doses of ethanol modulates
85 Heat Shock Protein (HSP) expressions in *Arabidopsis* seedlings. HSPs are molecules known to be
86 expressed in response to a wide range of stresses, including thermal, chemical, and oxidative stress
87 (Park and Seo, 2015). Among these HSPs, there is a subfamily called small heat shock proteins (sHSPs)
88 which is proposed to act as molecular chaperones to protect other proteins from stress-induced
89 damage (Waters and Vierling, 2020). The relationship between hypoxia and HSPs has already been
90 studied, and it has been shown that the activation of HSPs is critical to adaptation to hypoxia and for
91 enduring the oxidative stress of reoxygenation (Baird *et al.,* 2006). Chen *et al*., (2014) further showed
92 that HSP70 accumulated more than 10 folds in the soybean plasma membrane in response to hypoxia
93 stress. However, none of these studies established the link between ethanol, hypoxia and HSP.
94 Moreover, most studies on ethanol investigate organisms' response to "hammer" doses of this simple
95 alcohol, i.e. non-physiological doses.

96 In the present study, we investigate the impact of ethanol at a physiological dose, 1 mM, on
97 gene transcript expressions in grapevine cell cultures, as Xiao *et al*. (2018) have shown that hypoxic
98 conditions occurred during grape berry development. Since the late 1970s, it has been widely accepted
99 that plant cell cultures have numerous applications, among which serving as an excellent system for
100 studying plant cell genetics, physiology, biochemistry, and pathology (Smetanska, 2008). However,
101 there is a noticeable gap in the literature regarding the characterization of cell cultures grown on solid
102 media. Indeed, most research focuses on plant cell suspension cultures (Hellwig *et al*., 2004; Sato,
103 2013), rather than on plant cell cultures grown on agar-solidified medium in Petri dishes (i.e., calli).

After characterising the physiological endogenous concentrations of oxygen and ethanol in these cell cultures, we carried out a whole-genome transcriptomic study using RNA-seq. Among the gene families up-regulated by a physiological dose of ethanol treatment, we identified genes encoding for HSPs. Following this observation, a series of metabolite leakage measurements confirmed the protective role of ethanol priming on grapevine cells exposed to heat shock, initially shown by Matsui *et al*. (2022) in lettuce leaves.

Materials and methods

Plant materials

Experiments were performed on a grapevine model. Cells from the berry of *Vitis vinifera* L. cv. Gamay Fréaux were sub-cultured in 55x15 mm Petri dishes using a previously published method (Triantaphylidès *et al*., 1993), with modifications concerning the culture medium. The composition of the growth medium used in this study is given in Supplementary Table S1. Parafilm was wrapped around the plates to prevent the medium and the calli from drying out. The plates were then placed in a climatic chamber (CRYO RIVOIRE, France) under constant conditions with a day/night photoperiod of 16h/ 8h, a temperature of 25°C/ 20°C and a light intensity of 150 µmol.m-1.s-1. Plates were moved every day within the chamber to ensure homogenous growth conditions.

A complementary experiment was performed on *Arabidopsis thaliana* seedlings var. Col-0. Seeds were sterilised using a 4.8% bleach solution for 10 minutes. Following sterilisation, the seeds were sown individually onto 35x15 mm Petri dishes containing MS/2 medium with 0, 0.1 or 1 mM ethanol (EtOH), using a sterile toothpick for placement. The Petri dishes were then sealed with micropore surgical tape (3M, 1.25 cm width, Germany) and stratified at 4°C for 24 hours. After stratification, the plates were transferred to a growth chamber (CRYO RIVOIRE, France) where the seeds were cultivated for 11 days under controlled conditions: light/dark, 25/20°C, 14h/10h and 150 µmol.m-1.s-1. Plates were moved every day within the chamber to ensure homogenous growth conditions.

Characterization of cell cultures of *Vitis vinifera* L. cv. Gamay Fréaux.

<u>Callus development monitoring:</u> Calli were grown for five weeks and photographed at 0, 7, 14, 21, 28 and 35 days in Petri dishes. The selected calli shown in the results were representative of the observed general development. For each time point, the average callus weight has been measured using a microscale. Calli grown for 14 and 35 days were harvested for cross-sections and photographed using the Axio Zoom.V16 microscope (ZEISS, Germany).

139 Endogenous ethanol measurements: For different stages, calli were frozen in liquid nitrogen.
140 Each callus was ground to a fine powder and aliquoted at 200 mg/sample. The samples were then
141 thawed in an Eppendorf 5415R centrifuge (Eppendorf, Germany) at 4°C and 13,600 g for 10 minutes.
142 The supernatant was recovered and assayed for ethanol content using an enzymatic assay by
143 measuring NADH absorbance at 340 nm (Ethanol kit, BioSentec, France)
144 Endogenous oxygen measurements: Dissolved oxygen in grapevine calli was measured using a
145 Clark O_2 microelectrode with a 25 µm diameter tip (OX-25; Unisense A/S, Denmark). The
146 microelectrode was calibrated in 0% oxygen solution (0.1 M NaOH, 0.1 M $C_6H_7NaO_6$) and in fully
147 oxygenated water (273 µmol.L^{-1} at 22°C) as 100% O_2 solution. Individual callus (equilibrated to room
148 temperature) was placed on a motorised micromanipulator. The microelectrode was placed in the
149 middle of the callus and [O_2] profiles were taken in depth towards the centre of the callus at 300 µm
150 increments. Oxygen readings were recorded using Unisense Suite software (Unisense A/S).
151 Measurements were performed using 4 calli for each week of development.
152

153 ## EtOH treatment and sampling for RNA sequencing

154 We selected the callus stage (i.e. 21 days, Fig. 1A) to have sufficient weight of plant material
155 for the RNA extractions and because calli were in a healthy growing phase. Considering the
156 endogenous ethanol levels in the callus at 21 days (406 ± 2 µM, Fig. 1C), we chose to treat our calli
157 with 1 mM EtOH for the experiment. This treatment represents a 13% increase in ethanol
158 concentration based on average callus weight and the volume of the treatment solution (see
159 Supplementary Table S2). Due to the limited research on this subject, two sampling times were chosen:
160 6 hours and 24 hours post-treatment, to cover what we called short and long-term responses. Cell
161 cultures were thus treated with either 100 µL of a control solution (fresh growth medium) or with 100
162 µL of a fresh growth medium containing 1 mM EtOH; and then placed back in their culture chamber
163 for either 6 hours or 24 hours. Growth culture medium for this treatment purpose was prepared
164 without agar. After the incubation time, each callus was then collected in 10 mL Falcon™ tubes and
165 immediately immersed in liquid nitrogen, then stored at -80°C.
166

167 ## RNA extraction and purification

168 Frozen callus samples were homogenised to powder with the ball grinder Tissue Lyser II
169 (Qiagen, Germany) by applying one cycle of 2 minutes at 300 Hz. Total RNA from individual calli was
170 extracted using 160 mg of frozen powder with the Promega ReliaPrep™ RNA Tissue Miniprep System
171 kit (Promega, France, Ref. Z6112) following the Promega - ReliaPrep™ RNA Tissue Miniprep System
172 protocol, optimised for RNA extractions of grapevine cell cultures (see modifications in Supplementary
173 protocol). RNAs underwent DNAse treatment with the Invitrogen™ ezDNase™ Enzyme kit to remove

174 genomic DNA contamination from RNA preparations, following the ezDNase™ Enzyme kit protocol. For
175 each sample, quantity and purity were first assessed by measuring the optical density (OD) at 260 nm
176 and 280 nm with a NanoDrop® ND-1000 UV-Vis spectrophotometer (ThermoFisher, USA) and with a
177 1% (w/v) gel electrophoresis. Then, for each sample, RNA Integrity Number (RIN) was measured using
178 an Agilent 2100 Bioanalyzer System microfluidic on-chip capillary electrophoresis device, following the
179 RNA 6000 Nano kit protocol (Agilent Technologies, Germany).
180 Samples with a RIN > 8.8 were selected and then aliquoted to obtain 20 µL RNA samples at 50 ng.µL^{-1}.
181

182 ## RNA-seq analyses

183 Selected RNA samples were sent to Novogene Bioinformatics Technology Co., Ltd (Beijing,
184 China) for cDNA library construction and sequencing on the IlluminaNovaSeq 6000 platform (Illumina,
185 San Diego, CA, USA). For each condition, three biological replicates were sent. Raw sequences
186 corresponding to paired-end reads (2 x 150 bp) were treated as described by Althiab-Almasaud *et al*.
187 (2021) and Chirinos *et al*. (2023) but adapted for the grapevine genome. The raw data discussed in this
188 publication have been deposited in NCBI's Gene Expression Omnibus (Edgar *et al*., 2002) and are
189 accessible through GEO Series accession number GSE275842
190 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE275842). The reads were aligned on the
191 *Vitis vinifera* L. cv. Chasselas annotated reference genome produced by Djari *et al*. (2024). The quality
192 of these alignments is shown in Supplementary Tables S3A and S3B.
193

194 ## Differential Expression and downstream analyses

195 First, we normalised the counts according to the transcript lengths to compare transcript
196 expressions in downstream analyses. These normalised expressions can be understood as a relative
197 estimation of transcript counts. The mean value of the biological replicates was calculated, giving as a
198 result the estimated transcript counts for the given condition. Then, Differential Expression (DE)
199 analysis was performed as described by Chirinos *et al*. (2023) with R software version 4.1.1
200 (https://www.r-project.org/) using the DESeq2 package version 1.30.1 with the default relative log
201 expression (RLE) normalisation method, encompassing biases caused by library size and relative size
202 of transcriptomes (Maza *et al*., 2013; Love *et al*., 2014). More precisely, DE analyses were performed
203 as pairwise comparisons between all pairs of conditions (ex: “etha6h_1mM" versus "etha6h_0mM”).
204 The false discovery rate (FDR) was controlled by the Benjamini–Hochberg method with an adjusted p-
205 value (padj) < 0.05. Principal Component Analysis (PCA) was performed on estimated transcript counts
206 with the plotPCA function of DESeq2 after a variance stabilising transformation.
207
208

209 ## Identification and annotation of the differentially expressed genes (DEGs)

210 The functional annotation of the DEGs was retrieved from the VitExpress platform. When
211 functional annotations were missing, gene ID codes were converted from "Vv" to the taxonomic ID of
212 PNv4 "Vitvi" (Canaguier *et al*., 2017) using the GBFwebtool gene ID converter
213 (https://www.grape.resources.gbfwebtools.fr/converter). The "Vitvi" codes were then used to obtain
214 complementary annotations through MapMan Classification (https://mapman.gabipd.org/home)
215 (Schwacke *et al*., 2019). If both annotations were missing, the "Vv" code was used to extract the
216 corresponding gene sequence using the GBFwebtool "Extract Sequences"
217 (https://www.grape.resources.gbfwebtools.fr/extractSequences) and was subsequently blasted in
218 NCBI to find similar sequences and putative function (https://blast.ncbi.nlm.nih.gov/Blast.cgi).
219

220 ## Gene Ontology Enrichment analysis

221 Gene ontology (GO) enrichment analysis was performed with R using the enrichGO function
222 from clusterProfiler package version 4.10.1. Specifically, GO analysis was conducted separately on both
223 the up-regulated and down-regulated DEG lists obtained from the comparison between EtOH-treated
224 and control calli. Only DEGs with a |Log2FoldChange|>2 were selected.
225

226 ## EtOH priming and heat-shock treatments

227 For *Vitis vinifera* cv. Gamay Fréaux cell cultures: 200 µL of liquid growth medium with EtOH at
228 different concentrations (0, 0.1, 1, 10, 100 or 1000 mM) were poured onto three-week-old calli under
229 sterile conditions. Petri dishes were then sealed with micropore surgical tape (3 M, 1.25 cm width,
230 Germany) and returned to the culture chamber for 68 hours. After this incubation time, optimised by
231 preliminary experiments, the heat shock (HS) was applied for 15 minutes in an incubator in the dark,
232 with a peak temperature reaching 47°C (recordings of the heat stress peak can be found in
233 Supplementary Fig. S1.). The Petri dishes were allowed to cool down for 20 minutes before returning
234 to the growth chamber. Controls were treated with a control solution (liquid growth medium without
235 EtOH) and were subjected to heat stress. Double negative controls were treated with a control solution
236 (liquid growth medium without EtOH) and were not subjected to heat stress, but were kept in the dark
237 for the duration of HS.

238 For *Arabidopsis thaliana* HS treatment, the protocol was adapted from Matsui *et al*. (2022)
239 with the following modifications. *Arabidopsis* seedlings grown for 11 days with 0, 0.1 or 1 mM EtOH
240 were washed with deionized water and placed in test tubes containing 1.8 mL of distilled water (15
241 shoots/tube). The heat stress was applied by placing the tubes in a water bath at 43 °C for 30 minutes
242 and then cooling them down in a water bath set at 25 °C for 30 minutes. Control tubes did not undergo
243 HS and were kept in a 25 °C water bath for 1 hour.

Measurements of cellular leakage after HS treatment

To assess the cell damage caused by heat stress in *Vitis vinifera* and *Arabidopsis thaliana*, we selected cellular leakage as a key indicator of cell membrane integrity. Indeed, cellular leakage refers to the uncontrolled release of substances, such as ions, metabolites, or macromolecules, from the inside of a cell to its surrounding environment. This phenomenon typically occurs due to damage or disruption to the cell membrane (Jiang et *al.*, 2015), which normally serves as a selective barrier.

For *Vitis* cell cultures, cellular leakage measurements were assessed by measuring the coloured compounds released into the callus rinsing solution. The protocol was adapted from Ilík *et al.* (2018) with the following modifications: 72 hours after HS, calli were harvested, placed in 2 mL Eppendorf tubes, and weighed. To each tube, 1 mL of 100 mM mannitol rinsing solution was added to limit osmotic shocks. The tubes were then vortexed for 15 seconds and incubated at room temperature for 20 minutes on a rotator wheel, followed by centrifugation for 2 minutes at 16,100g. The absorbance of the supernatant was measured at wavelengths of 420 nm, 520 nm and 620 nm (Chervin *et al*., 2001; Lambri *et al*., 2015). The 100 mM mannitol solution was used as a blank.

We reported the results as relative absorbances, normalised to the HS controls (absorbance values set to 1) to account for variability between different batches.

For *Arabidopsis* seedlings, cellular leakage was assessed by the Index of Injury (Id) (Ilík *et al.*, 2018), calculated from electrical conductivity measurements. The Id is a quantitative measure used to assess the extent of damage in plant tissues in response to stress conditions such as temperature extremes. It provides an indication of the degree of cellular or tissue damage, and evaluates the impact of various stressors on plant health. Based on a protocol adapted from Matsui *et al*. (2022), *Arabidopsis* seedlings grown for 11 days were washed with deionized water and placed in test tubes containing 1.8 mL of distilled water (15 shoots/tube). The heat stress was applied as described previously. Heat-stressed and control samples were slowly shaken overnight at 30 rpm, after which electrical conductivity was measured with a conductivity metre (Yangers, UK). The samples were then heated for 1h in a 100 °C water bath and shaken at room temperature for several hours, after which the electrical conductivity of the samples was again measured. The index of injury (Id) was calculated based on the protocol described for ion leakage analysis of *Arabidopsis seedlings* by Ilík *et al*. (2018).

Anthocyanin content determination

This protocol was adapted from Vitrac *et al*. (2000), which investigated cell suspensions of *Vitis vinifera* (L.) and more notably those of cv. Gamay Fréaux Teinturier. Some modifications have been made: Three-week-old calli were treated with 100 µL of either a 1 mM EtOH or a control solution and were incubated for up to 16 days post-treatment in their growth chamber. Fresh callus tissues were

279 collected, weighed, and placed into 1.5 mL Eppendorf tubes. Each tube received 500 µL of a solvent
280 mixture composed of methanol and 0.32M HCl (85:15, v/v). The tissues were ground with a tube pestle
281 for 1 minute, followed by incubation for 2 hours at 4 °C on a rotator wheel. After incubation, the tubes
282 were centrifuged at 16,000g for 2 minutes. The supernatant was diluted to 1/50th of its original volume
283 with the same solvent.
284 The absorbance of the anthocyanin extract was measured at 535 nm. Anthocyanin content was
285 calculated using the molar extinction coefficient (log ε = 4.53) as reported by Vitrac *et al*. (2000).
286 Methanol and 0.32M HCl (85:15, v/v) solvent was used as a blank.
287

288 ## Total Polyphenol Index determination

289 The Total Polyphenol Index (TPI) is a measure used to quantify the total amount of
290 polyphenolic compounds present in a sample. The TPI method relies on the absorption of benzene
291 rings at 280 nm, a characteristic feature of polyphenols. This protocol was adapted from Cetó *et al*.
292 (2012) with the following modification: on the same supernatant extracts as those prepared for
293 anthocyanin determination, absorbance was measured at 280 nm using a quartz cuvette. The TPI for
294 each sample was calculated as the absorbance multiplied by the appropriate dilution factor. Methanol
295 and 0.32M HCl (85:15, v/v) solvent was used as a blank.
296

297 ## Statistical analyses

298 All data in Figures 1 and 4 were analysed by ANOVA and multiple comparison tests, performed
299 with Sigmaplot v.15 (Inpixon, USA). The confidence interval was set at 95% and the p-value is set at
300 0.05. Significantly different groups are indicated by small letters (a, b, c) on the graphs.
301 For RNA-seq data, the statistical analyses were performed using the R software, as specified in the
302 previous paragraphs.
303

304 # Results

305

306 ## Grapevine *Vitis vinifera* L. cv. Gamay Fréaux cell cultures are characterised by
307 hypoxia and physiological ethanol levels in the µM to mM range

308 Figure 1A shows pictures of grapevine cell cultures as a function of development time over five
309 weeks. The increase in callus size throughout their development is noticeable, but between days 28
310 and 35 of development, callus size varies only slightly (average callus weight over 5-week-development
311 can be found in Supplementary Fig. S2). Furthermore, the typical colour of our study model, a bright
312 pink to light red, is evident in the first four weeks of development. A noticeable colour change occurs

313 between the 28th and 35th days of development: by the 35th day, the calli appear duller compared to
314 those grown for 0, 7, 14, 21, and 28 days.

315 We then characterised the levels of oxygen in the grapevine calli during five weeks of
316 development on growth media. Figure 1B shows dissolved O_2 levels at a distance of 2100 µm towards
317 the callus centre as a function of development time. We found that O_2 levels increase substantially and
318 gradually over time of development, starting with concentrations of around 10 µmol.L^{-1} in 7-day-old
319 calli to 150 µmol.L-1 in 35-day-old calli. Dissolved oxygen levels in calli grown for 7 and 14 days were
320 statistically lower than in 35-day-old calli. Complete O_2 profiles inside calli were measured and sections
321 of 14-day-old and 35-day-old calli were observed, and results are shown and detailed in Supplementary
322 Figure S3, and Supplementary Figure S4, respectively. Regarding the natural endogenous levels of
323 ethanol in calli, we found a strong decrease in ethanol concentration as a function of development
324 time, with an inverse relationship to oxygen levels (Fig. 1C). Ethanol concentration was significantly
325 higher in 7-day-old calli than in 35-day-old calli, decreasing from 1726 ± 190 µM to 121 ± 99 µM,
326 respectively.

327 Therefore, we can define the range from 100 µM to 1700 µM as the "physiological" ethanol
328 concentrations present in cell cultures of *Vitis vinifera* L. cv. Gamay Fréaux under normal growth
329 conditions.

330

331 A small exogenous EtOH dose impacts the grapevine transcriptome

332 To investigate the impact of a physiological dose of EtOH on the *Vitis vinifera* transcriptome,
333 we employed RNA sequencing (RNA-seq). Overviewing the transcriptional dynamics of the RNA-seq
334 experiment, the Principal Component Analysis (Fig. 2A) revealed significant distinctions among the
335 generated transcriptomes, with 74% of the variance associated with PC1 and 14% with PC2. The data
336 consistently demonstrates a clustering pattern, wherein transcriptomes align closely according to their
337 respective treatments, forming 4 distinctive clusters (0mM_6h, 1mM_6h, 0mM_24h, and 1mM_24h).
338 This showed that biological variability was smaller than the differences between the four conditions
339 and suggests a discernible impact of a low 1 mM dose of EtOH on the transcriptomes. There is more
340 distance between the control and ethanol treatment at 6 hours than at 24 hours (Fig. 2A). Additionally,
341 it is noteworthy that the 1mM EtOH treatment at 6 hours and at 24 hours show the greatest distance
342 in the PCA plot, whereas the control solution at 6 hours and 24 hours generated the closest
343 transcriptome groups. There were 5527 DEGs in the 6 hours comparison whereas there were 3755
344 DEGs in the 24 hours comparison (Supplementary Fig. S5).

345 To further refine our analysis, we applied more stringent criteria to DEGs: we selected genes
346 with a |Log2FoldChange|>2, in addition to requiring an adjusted p-value < 0.05 (raw results of the
347 Differential Expression Analysis of the comparison 1 vs 0 mM EtOH at 6 hours are available in

348 Supplementary Table S4). For the comparisons at both 6 and 24 hours, the DEG analysis revealed that
349 there was a greater number of down-regulated DEGs (Fig. 2C, D). These results show a transcriptional
350 trend towards inhibition, with most genes under-expressed in ethanol-treated calli. When visually
351 comparing the number of DEGs between the two post-treatment durations, the volcano plot for the
352 6-hour time point shows a greater number of overall DEGs than the plot for the 24-hour comparison
353 (386 vs. 106 genes respectively, see Fig. 2D).
354 These results indicate that even a light change in ethanol significantly impacts the grapevine
355 transcriptome.
356

357 EtOH treatment within the physiological range significantly upregulates small 358 heat shock protein-related gene expressions

359 Considering the higher number of DEGs observed at 6 hours compared to 24 hours post-
360 treatment, we decided to focus on the responses 6 hours after treatment. The complete lists of the
361 386 functionally annotated DEGs is available in Supplementary Table S5, sorted out in two sublists:
362 Supplementary Table S5A provides the down-regulated DEGs (254 genes) and Supplementary
363 Table S5B provides up-regulated DEGs (132 genes). Figure 3A shows the results of this GO enrichment
364 for the up-regulated DEGs (p-adj < 0.05 and a Log2FoldChange > 2) while Figure 3B shows the GO
365 enrichment for the down-regulated DEGs (p-adj < 0.05 and a Log2FoldChange < - 2) (the overall GO
366 analysis results can be found in Supplementary Tables S6 and S7 respectively).
367 We found that genes annotated as “response to heat” (GO:0009408) and “response to temperature
368 stimulus” (GO:0009266), “protein self-association” (GO:0043621) and "unfolded protein binding"
369 (GO:0051082) were the most represented with a gene ratio of 0.158, among the 132 up-regulated
370 DEGs (Figure 3A). For down-regulated genes, we found that genes annotated as “secondary
371 metabolism process” (GO:0019748) and “secondary metabolite biosynthetic process” (GO:0044550)
372 were the most represented among the 254 down-regulated DEGs, with gene ratios of 0.08 and 0.07
373 respectively (Fig. 3B).
374 Given these results, we had to examine the specific genes comprising the enriched families in
375 both DEGs lists. First, we examined the specific genes comprising the up-regulated enriched family
376 associated with the GO term "Response to heat". This list includes 15 DEGs, presented in Figure 3C
377 (their respective gene IDs, functional annotations, Log2FoldChange values, and adjusted p-values can
378 be found in Supplementary Table S8). When looking at the functional annotations in Figure 3C, all the
379 up-regulated DEGs are small Heat Shock Proteins (sHSP) and they are sorted out in two major groups:
380 9 genes are cytosolic class I sHSP and 5 genes are cytosolic class II sHSP. The last gene is a class-P sHSP.
381 The Log2FoldChanges of these DEGs range from 2.09 to 7.09 and their associated p-adj values are very
382 low (0.039 to 5.19^{-12}), providing a high level of confidence that the ethanol treatment is responsible
383 for this differential expression.

384 Then, we examined the specific genes comprising the down-regulated enriched family associated with
385 the GO term "Secondary metabolism process". This list includes 16 DEGs, presented in Supplementary
386 Figure S6 (their respective gene IDs, functional annotations, Log2FoldChange values, and adjusted p-
387 values can be found in Supplementary Table S9). When looking at the functional annotations in
388 Supplementary Figure S6 we can notice genes involved in the general phenylpropanoid pathway like
389 the pivotal phenylalanine ammonia-lyase (PAL), and genes involved in phenylpropanoid down-stream
390 pathways. Indeed, genes involved in pathways for stilbenoid and flavonoid biosynthesis, such as
391 stilbene synthase (STS) and chalcone synthase (CHS) are down-regulated. The Log2FoldChanges
392 associated with these DEGs range from -2.02 to 4.90, being less extremes than the ones associated
393 with the “Response to heat” genes.
394

395 ## Ethanol-priming reduced heat-induced cell leakage

396 Following the observation of significant and highly up-regulated sHSPs revealed in the GO
397 analysis, we looked for thermotolerance phenotypes in ethanol-primed calli after heat stress.
398 The results are detailed in Figure 4. We observed that the double negative controls, which entails
399 neither ethanol priming nor exposure to heat stress, exhibited the lowest absorbances at 420, 520 and
400 620 nm respectively (cyan bars, Fig. 4A-C), showing the basal cellular leakage in our experimental
401 conditions. In contrast, cellular cultures exposed to both the control pre-treatment (0 mM EtOH) and
402 HS demonstrated the highest absorbance values at all three wavelengths. Calli primed with EtOH
403 solutions ranging from 0.1 to 10 mM, displayed a continuous decreasing trend in the absorbance values
404 measured at 420 nm, 520 nm, and 620 nm, compared to the controls. The absorbances increased again
405 within calli primed with 100 and 1000 mM EtOH solutions. These particular inverted bell-shaped curves
406 are observed across all three monitored absorbances.
407 Overall, calli primed with concentrations of 1 and 10 mM showed strong reductions in
408 absorbance around 35-45% compared with the HS-control. These results show that priming grapevine
409 cellular cultures with low physiological doses of ethanol (i.e. 0.1 to 10 mM) reduced the cellular
410 compound leakage after exposure to a mild heat shock. Moreover, a complementary experiment was
411 conducted on *Arabidopsis thaliana* seedlings to confirm that low doses of EtOH could limit cellular
412 leakage in another plant’s tissues and in a different plant species. Figure 4D presents ion leakage for
413 *Arabidopsis thaliana* seedlings. We found that in control seedlings, the injury index at 83% was
414 significantly higher than in seedlings treated with 0.1 and 1 mM EtOH, which were at 69% and 75%
415 respectively. This corresponds to a significant reduction in the injury index by 14% for the 0.1 mM
416 ethanol treatment and 8% for the 1 mM ethanol treatment compared to control. This result shows
417 that priming *Arabidopsis* seedlings with low physiological doses of ethanol (i.e. 0.1 and 1 mM)

418 significantly reduced the ions released after exposure to a mild heat shock, and further reinforces the
419 results described for the Gamay cell cultures.
420

421 Ethanol-priming does not impact polyphenol content in grapevine calli

422 As shown in Figure 3B, the GO terms 'Secondary metabolic process' and 'secondary metabolite
423 biosynthetic process' are significantly enriched among the 254 down-regulated DEGs, many of which
424 are linked to polyphenol synthesis (see Supplementary Fig. S6). Given this significant down-regulation
425 observed in secondary metabolism, and particularly the down-regulated PAL and CHS transcripts, we
426 investigated polyphenol content in ethanol-primed calli. To assess this further, we monitored
427 anthocyanin and total polyphenol content as a function of time in grapevine cell cultures, following an
428 initial 1 mM EtOH treatment. The supplementary Figure S7 illustrates the changes in anthocyanin
429 concentration and total polyphenol index in cell extracts. Both graphs show an increasing trend as a
430 function of time, however, there was no significant difference in anthocyanin concentrations
431 (Supplementary Fig. S7A) or in the total polyphenol index (Supplementary Fig. S7B) between the
432 ethanol-primed and control treatments.
433
434

435 Discussion

436 Grapevine cell cultures, a model experiencing hypoxia and ethanol accumulation

437 A recent study by Xiao *et al (*2018) showed low levels of oxygen in grape berries and even
438 anoxia in the centre of the berries. Our grapevine cell culture model shows the same trend, with
439 developmental hypoxic to anoxic oxygen levels and inversely correlated ethanol concentrations in the
440 calli, especially when they undergo rapid growth in the first 2 weeks after subculturing (Fig. 1B,C). This
441 rapid growth is associated with a high rate of cell division and high cellular oxygen consumption in
442 most plant tissues (Raymond *et al*., 1995). The increase in oxygen levels in calli, as they age, is probably
443 linked to a decrease in their growth and metabolic rates. This slower growth allows oxygen to diffuse
444 faster than it is consumed by the cells. Additionally, after five weeks of growth, cross-sections
445 (Supplementary Fig. S4) revealed a heterogeneous structure with invaginations and gaseous interstices
446 within the callus, further facilitating air and oxygen diffusion. A close relationship exists between
447 oxygen and ethanol levels in plants, in which decreased oxygen levels result in increased ethanol
448 concentrations, due to the shift from aerobic to anaerobic respiration (Bui *et al*., 2019). Thus, as
449 dissolved oxygen levels increase over callus developmental time, it is logical to observe a
450 corresponding decrease in ethanol levels. Therefore, we can conclude that our cell culture model
451 naturally experiences developmental hypoxia, leading to ethanol accumulation. This validates the cell

452 culture model for studying the impact of small changes in ethanol concentrations. These latter could
453 be related to climate change, as rising temperatures in this context further reduce dissolved oxygen
454 levels in water and organisms, while increasing respiration rates. These changes lead to hypoxic
455 metabolism in certain organisms and tissues, and to ethanol production. The question arises whether
456 ethanol serves as a signal prompting organisms to adapt, or if it is simply an end-product of hypoxic
457 metabolism (Diot *et al*., 2024).

458

459 Small exogenous application of EtOH triggers a fast response in the grapevine 460 transcriptome and cells seem to perceive it as a stress.

461 We present evidence that small changes in ethanol concentration generate quick responses at
462 the transcriptomic level in Gamay cell cultures. On the PCA plot (Fig. 2A), the closely grouped
463 transcriptomes based on their respective treatments and the high explained variance demonstrate the
464 significant impact of ethanol on gene transcripts. The sum of the variances (PC1 + PC2), which accounts
465 for 88% of the total variance, indicates that the two-dimensional plot effectively represents the impact
466 of the ethanol treatment and the two sampling time points (6 and 24 hours). It is possible that the
467 highly controlled *in vitro* culture environment and the use of identical cloned cells limit the biological
468 variability in our experimental design. The fact that Gamay cell cultures can sense very small changes
469 in ethanol concentration (1 mM) is evidenced by the 386 DEGs identified at 6 hours (Fig. 2D),
470 suggesting that a small amount of ethanol is quickly detected by the grapevine cells and prompts a
471 strong and immediate transcriptional response. The reduction in the number of DEGs to 106 at 24
472 hours post-treatment (Fig. 2D) shows that the transcriptional response diminished over time. This
473 result is further reinforced by the decreased number of DEGs with a high Log2FoldChange after 24
474 hours compared to 6 hours (Fig. 2C versus Fig. 2B). This trend suggests that the transcriptional
475 response to low doses of ethanol is not only strong and immediate but also relatively transient. The
476 general trend of downregulation of the transcriptomic dynamics when comparing 1 mM EtOH to
477 control treatments (Fig. 2D), at 6 and 24 hours post-treatment, is often observed in plant responses to
478 stresses. Indeed, Ul Haq *et al.* (2019) showed that a plant's common strategy to cope with stressful
479 situations is reducing the synthesis of normal proteins, while enhancing the production of stress-
480 related proteins, such as HSPs. This trade-off is clearly illustrated at the transcriptomic level in our
481 study: Gamay calli downregulate genes involved in secondary metabolism (Fig. 3B) while
482 simultaneously overexpressing a set of genes related to stress proteins following the addition of
483 exogenous ethanol (Fig. 3A). This GO analysis revealed that cell sHSP genes were upregulated in calli
484 treated with 1 mM EtOH compared to the control at 6 hours (Fig. 3A). sHSPs function as early
485 responders to assist cells in managing proteins destabilized by stress conditions (Janowska *et al*., 2019),
486 and their induction represents a rapid and intense emergency response (Parsell and Lindquist, 1993).

487 This finding aligns with our RNA-seq data, as we observed that, just 6 hours after treatment with 1 mM
488 EtOH, some sHSP genes were expressed more than 64 times than in the control (Fig. 3C). Our study
489 suggests that a minor change in ethanol is sensed by the cells as a stress, prompting them to initiate
490 adaptive responses.

491

492 Responses of grapevine cells to physiological doses of ethanol: upregulation of 493 small heat shock protein transcripts.

494 Our results show that the genes associated with the GO terms "Response to heat" and
495 "Response to temperature stimulus" are the most statistically overrepresented 6 hours after a 1mM
496 EtOH treatment, notably the sHSPs (Fig. 3C).
497 Firstly, the ability of ethanol at physiological doses to stimulate sHSP synthesis has already been
498 observed in the animal kingdom. Indeed, some studies showed that ethanol at 50 mM induced sHSP
499 genes in mouse embryonic neural stem cells (Choi *et al*., 2011) and EtOH at 60 mM increased the levels
500 of HSP27 in mouse cortical neurons (Pignataro *et al*., 2007).
501 <u>sHSPs:</u> HSPs are classified into five major subfamilies based on their molecular weights:
502 Hsp100, Hsp90, Hsp70, Hsp60, and small heat shock proteins (sHSPs) (Sun and MacRae, 2005). These
503 latter are differentiated from the other HSPs by their smaller molecular weight -typically, plant sHSPs
504 range between 15 and 30 kDa-, and are the most abundant in plants (Vierling, 1991). The sHSPs are
505 expressed during the normal developmental process of plants (Lindquist and Craig, 1988), and are
506 known for their response to a wide range of environmental stresses, including heat, cold, drought,
507 salinity, and oxidative stress; and it seems that the high diversification of plant sHSPs reflects a
508 molecular adaptation to stress conditions. As it is clear that sHSPs have evolved to be sensitive to small
509 changes in pH or in metal ions near physiological concentrations (Janowska *et al*., 2019), it would be
510 logical that they are also sensitive to other small changes near-physiological state like, in our case,
511 small changes in ethanol concentration. The small HSPs are particularly renowned for their crucial role
512 in maintaining proteostasis when expressed in response to cellular stress in plant systems (Haslbeck
513 and Vierling, 2015). Indeed, they specialized in preventing protein aggregation by binding to partially
514 unfolded proteins under stress conditions (McLoughlin *et al*, 2016; Janowska *et al*., 2019; Lee *et al*.,
515 1995, 1997), differentiating from other larger HSPs in their role, as sHSPs do not typically refold
516 proteins. Besides this activity, sHSPs can function as membrane stabilisers and ROS scavengers or act
517 synergistically with antioxidant systems. Overall, they play a key role in maintaining membrane fluidity
518 and permeability under some stress (Aghdam *et al*., 2013). However, ethanol was shown to modify the
519 fluidity of phospholipid bilayers (Patra *et al*., 2006), possibly creating a link between ethanol,
520 membrane organization, and the induction of sHSPs.

521 Class CI and CII sHSPs: In Figure 3C, the key genes attracting our attention were sHSP genes
522 encoding for class CI and CII subfamilies. These are the two most abundant classes of plant cytosolic-
523 localised sHSPs. CI sHSPs typically comprise the largest family of sHSP genes in higher plants (Waters,
524 2013) while the CII gene family is typically smaller. Basha *et al*. (2010) examined the structural and
525 functional differences between the CI and CII subfamilies. They reported that both the CI and CII
526 subfamilies have chaperone activities and they were effective at preventing irreversible aggregation,
527 illustrating their role in the prevention of future damages made to the cell. Therefore, it is not so
528 surprising that 6 hours after the EtOH treatment, we observed a significant up-regulation of these
529 protein transcripts. Thus, we can hypothesize that the plant may use very small changes in ethanol to
530 prevent further damage due to stresses. Indeed, ethanol has been shown to induce sHSP in soybean
531 (Kuo *et al*., 2000) and in rice (Guan *et al.*, 2004), even if the ethanol doses tested were 5% (v/v)
532 corresponding to a concentration of nearly 1M, which can be considered like a "hammer" dose.
533

534 Small changes in ethanol concentration confer thermotolerance to grapevine 535 calli by reducing cellular leakage, a trend confirmed by experiments with 536 *Arabidopsis* seedlings

537 Given that our plant model is a culture of cells rich in anthocyanins (Guan *et al*., 2016; Kong *et*
538 *al*., 2021), we drew inspiration from studies demonstrating the release of pigments following heat
539 stress (Lambri *et al*., 2015). Zhang *et al*. (2005) showed that heat treatment (38 °C for 10 h) induced
540 injuries in the ultrastructure of *Vitis* mesophyll cells, then linked to increase in conductivity associated
541 with membrane leakage, and also linked to malondialdehyde increase. In oenology, the postharvest
542 heat treatment of grape berries has been known for a long time to increase berry skin cell damage and
543 facilitate the release of anthocyanins (Lambri *et al*., 2015). These latter authors showed that heating
544 grape berries at various temperatures from 60 to 80°C for various times 3 to 60 min, increased the
545 absorbances at the main three wavelengths defining red wine colour intensity: 420, 520 and 620 nm,
546 the main red pigments being anthocyanins (Lambri *et al*., 2015). In our study, after preliminary
547 experiments, we chose a rather mild heat treatment (Supplementary Fig. S1) in order to illustrate
548 differences induced by ethanol priming. In our Figure 4A, 4B and 4C, significant differences in pigment
549 leakage indicate that ethanol priming protected grapevine calli from damage due to exposure to heat
550 stress, compared to control ("0"). This protective effect was also shown in a different plant species.
551 Indeed, significant differences in electrical conductivity indicate that ethanol priming also protected
552 *Arabidopsis* seedlings from damage due to exposure to heat stress (Fig. 4D), compared to control ("0").
553 In other words, ethanol priming effectively reduced cell leakage caused by heat treatment,
554 demonstrating that a low dose of ethanol enhances tolerance to heat stress, and suggesting that the
555 ethanol-induced-sHSPs are related to the acquisition of thermotolerance. Increasing data suggest a
556 strong correlation between sHSP accumulation and plant tolerance to stress (Liu *et al*., 2012; Wang *et*

557 *al*., 2004), like our data. These latter authors suggest that the protective effect involves membrane
558 stabilization. According to Ilík *et al.* (2018), heat acclimation is connected to an increase in the degree
559 of saturation of fatty acids of membrane lipids, which in turn leads to increased rigidity of cell
560 membranes and increase in their thermostability. A search for desaturase-related-genes in our RNA-
561 seq data did not bring evidence that ethanol priming is regulating this type of enzyme, at 6 or 24 hours
562 post-treatment. Nevertheless, Tsvetkova *et al*. (2002) showed that association between sHSPs and
563 membranes may constitute a general mechanism that preserves membrane integrity during thermal
564 fluctuations.

565 Plants have evolved many mechanisms for adaptation to dynamic and adverse environmental
566 conditions. In addition, they have acquired a form of 'stress memory' in that they retain their response
567 to an initial stress, which primes their response to a second stress, allowing them to respond more
568 rapidly and strongly (Savvides *et al*., 2016). One adaptation is their ability to produce a variety of
569 compounds that induce the expression of stress-related genes, whose encoded products protect
570 cellular components from the adverse impact of abiotic stress (Sako *et al*., 2020). This phenomenon,
571 called chemical priming, is evident in our results, as cell cultures that underwent ethanol priming
572 showed better protection against mild heat stress. Todaka *et al*. (2024) demonstrated that a 20 mM
573 ethanol treatment enhanced heat stress tolerance in tomatoes, and highlighted the potential of
574 ethanol-based chemical priming as a technology to mitigate heat stress damage in crops. Crosstalk
575 between heat and ethanol stresses, and heat and ethanol tolerance, has already been shown in several
576 species and seems to be a conserved trait across kingdoms. In plants, this has been shown by Matsui
577 *et al*. (2022). And in other kingdoms, HSP induction correlates with induced tolerance to high
578 concentrations of ethanol: in mammals, Chinese hamster (Li, 1983); in microorganisms,
579 *Saccharomyces cerevisiae* (Plesset *et a*l., 1982).

580 One point left to discuss is the timeframe of the responses. The sHSPs expression is often part
581 of an immediate stress response (Hu *et al*., 2022) but their synthesis is not maintained through time
582 (Altschuler and Mascarenhas, 1982). Our results reflect this, as no sHSP genes were up-regulated 24
583 hours post-ethanol treatment when compared to control. However, 68 hours after ethanol priming,
584 thermotolerance phenotypes were observed (Fig. 4A-C). Vierling (1991) reported that pea plants
585 exposed to heat stress accumulated chloroplastic sHSPs and class CI HSPs, even after the stress had
586 ended. The half-life of these proteins was found to be around 52 hours for chloroplastic sHSPs and 37
587 hours for cytoplasmic HSPs. This shows that although the expression of small heat shock proteins is
588 transient, the sHSPs themselves are quite stable. The persistence of these HSPs suggests that their
589 participation in recovery processes could be as important as their possible role during the stress period
590 itself (Vierling, 1991). However, the authors of the present study have to report that priming is
591 effective in mitigating heat stress when the stress is mild. Indeed, when the stress exceeds a certain

592 threshold (higher than sublethal heat shock), the priming effect diminishes, and the treated cells and
593 *Arabidopsis* seedlings exhibit worse leakage compared to the control (data not shown).
594
595

596 Responses of grapevine cells to physiological doses of ethanol: downregulation 597 of key polyphenol-related transcripts, without effects on anthocyanin and 598 polyphenol contents

599 As previously discussed, the general trend in the downregulation of secondary metabolism is
600 a classical plant response to stresses (Ul Haq *et al.*, 2019; Bilgin *et al*., 2010; Liu *et al*., 2012). However,
601 no significant difference was observed between EtOH treated and untreated calli regarding the
602 content of anthocyanins and the content of total polyphenols (Supplementary Fig. S7). We have two
603 main hypotheses to explain the difference between the transcriptomic and the physiological levels.
604 First, it could be that because the anthocyanin and polyphenol contents are so important in Gamay
605 Fréaux calli, that the transient downregulation of the enzymes involved in the biosynthesis of
606 polyphenols results in a too small physiological change. Indeed, the Gamay Fréaux is a teinturier grape
607 and stands out among grape cultivars due to its rare and unique red-fleshed berries as well as red skin,
608 which is uncommon compared to the majority of grape cultivars having red skin colour but white-
609 coloured flesh (Guan *et al*., 2016). Second, the polyphenol pathway is complex with numerous proteins
610 involved. The fact that the expression of some of these protein transcripts is inhibited does not ensure
611 that polyphenolic compounds accumulate at different levels.
612

613 Plant response to ethanol displays hormesis characteristics and ethanol could be 614 a potential messenger of hypoxia and other stressful conditions.

615 Because we saw the sHSPs subfamily being extremely overexpressed in the RNA-seq
616 experiment and in order to examine a physiological response of grapevine cell cultures to low doses of
617 ethanol, we assessed the cell damages resulting from a heat shock in calli primed with ethanol at
618 different concentrations. The results (Fig. 4A-C) show a clear biphasic dose-response curve for the
619 absorbances tested, typical of an hormesis effect. Hormesis is a phenomenon observed in biology
620 where exposure to low doses of a stressor or toxin results in a beneficial effect on an organism,
621 contrary to the expected harmful effect at higher doses (Calabrese and Baldwin, 2002; Calabrese and
622 Baldwin, 2003). This concept challenges the traditional linear dose-response model by proposing U-
623 shaped or inverse U-shaped response curves (Erofeeva, 2022). According to Calabrese and Baldwin
624 (2002; 2003), hormesis is considered an adaptive response of organisms to stimuli (environment,
625 chemicals, …). At low doses, the stressor triggers adaptive responses within cells and organisms, such
626 as increased antioxidant production (Wang *et al*., 2023) and repair mechanisms (Scott, 2008), which

627 enhance resilience and improve overall health. Calabrese and Baldwin (2002; 2003) suggest that this
628 stimulatory response could be the result of compensatory biological processes, following an initial
629 disruption in homeostasis, i. e. in our study, the slight change in ethanol concentration. Although there
630 is no single biological mechanism that can explain hormesis across plants, Agathokleous *et al*. (2020)
631 suggest that certain general principles regarding the mechanisms of hormesis across different species
632 and stress-inducing agents can be identified. These authors note that low doses of stress induce a mild
633 increase in the production of reactive chemical species, hormones, and enzymatic and nonenzymatic
634 antioxidants, as well as upregulation of proteins (e.g., heat shock proteins); and that these responses
635 lead to reduced toxicity if plants are subsequently exposed to severe stress, within a preconditioning
636 framework. Our study shows both an increase in heat shock proteins transcripts (Fig. 3B) and reduced
637 HS toxicity (Fig. 4) after an ethanol treatment. This hormesis effect of ethanol has already been seen
638 in a study by Middleton *et al* (1978) on its impact on the architecture of pea roots, where the mean
639 root length was increased by an ethanol concentration as low as 0.002 $ml.L^{-1}$ (equivalent to 0.0002%
640 or 0.034 mM). Moreover, this positive impact of low doses of ethanol has been observed and
641 confirmed by more recent studies. Indeed, Bhattacharya *et al*. (1985) showed that ethanol increased
642 the root formation in mung bean, with a maximum reached at 0.2% (equivalent to 34.25 mM), and Wu
643 *et a*l. (2019) reported that ethanol improved both biomass (at 0.0125 - 0.05 $mL.L^{-1}$, equivalent to 0.21
644 - 0.86 mM) and micronutrient accumulation in oilseed rape. The characteristic response of hormesis
645 can be observed in the results of transpiration of Gamay cuttings subjected to water stress and EtOH
646 priming, in a companion article published in this issue (Ait Kaci *et al*., 2024). And finally, the curve
647 shapes of cellular leakage observed in our study (Fig. 4), on two different plant species, strengthen the
648 previous results. Thus, our results suggest that ethanol at physiological concentrations interacts with
649 grapevine and *Arabidopsis* biological systems in a complex manner, where small amounts can activate
650 adaptive and beneficial responses, while larger amounts overwhelm these systems, leading to toxicity.
651 Given its potential role in activating adaptive responses, we hypothesise that ethanol may serve as a
652 signalling molecule in plants and could be a potential messenger of stressful conditions.
653 Further research is needed to explore ethanol-induced hormesis in plants, specifically to
654 elucidate how stimulation and inhibition vary with ethanol concentration. While the inhibition
655 observed at high concentrations is expected as it aligns with the onset of toxicity, the stimulation effect
656 at low concentrations warrants more detailed investigation. Considering ethanol status as a small
657 molecule with significant diffusive capabilities, notably through membranes, and considering its
658 hydrophobic nature, the manner by which cells perceive small variation in ethanol concentration is still
659 a matter of research and debate (Diot *et al*., 2024).
660
661

662 ## Conclusion

663 Our study demonstrated that grapevine cells sense ethanol at a physiological concentration,
664 inducing a strong and rapid transcriptomic response, typical of stressors. This transcriptomic response
665 had physiological repercussions, as evidenced by the thermotolerance biphasic dose-response curves,
666 suggesting that ethanol could act as a potential molecular messenger in stressful situations in plant
667 systems, rather than just being an end-product metabolite of hypoxic metabolisms. Harnessing this
668 response could be a promising strategy to prepare plants for more challenging stress conditions.
669 Indeed, plants biosynthesize various compounds, such as phytohormones and other metabolites, to
670 adapt to adverse environments. Recent studies have demonstrated that exogenous treatment of
671 plants with chemical compounds can enhance abiotic stress tolerance by inducing molecular and
672 physiological defence mechanisms, a process known as chemical priming (Sako *et al*., 2020). The
673 development of effective strategies that mitigate abiotic stress is essential for sustainable agriculture
674 and food security, especially with continuing global population growth. Chemical priming is believed
675 to represent a promising strategy for mitigating abiotic stress in crop plants. Given its low cost and
676 environmental non-persistence, we propose that ethanol could be a good candidate for plant chemical
677 priming and a valuable tool in agriculture, especially for mitigating environmental stress impacts on
678 crops.

679

680

681 ## Supplementary data

682

683 The following supplementary data are available at JXB online.

684

685 <u>List of Supplementary Figures</u>

686 **Fig. S1.** Heat-stress monitoring in Gamay cell cultures.

687 **Fig. S2.** Average weight of a callus of Vitis vinifera cv. Gamay as a function of development time
688 after sub-culturing.

689 **Fig. S3.** Dissolved O_2 profiles of Vitis vinifera L. cv. Gamay Fréaux cell cultures as a function of
690 development time.

691 **Fig. S4.** Photographs of calli seen from above and photographs of calli cross-sections.

692 **Fig. S5.** Transcriptional dynamics showing the number of DEGs upregulated and downregulated, 6
693 and 24 hours after a 1 mM ethanol treatment, compared to the controls.

694 **Fig. S6.** Details of the 16 DEGs within the enriched GO term "Secondary metabolism process".

695 **Fig. S7A and S7B.** Monitoring anthocyanin concentration and total polyphenol index of Gamay cell
696 cultures after an initial 1 mM ethanol treatment or control treatment.

List of Supplementary Tables

Table S1. Composition of the Gamay cell culture growth medium

Table S2. Table showing the 'concentration-effect' of the ethanol treatment in 3-week-old calli, at the time of treatment with the exogenous ethanol solution.

Table S3A and S3B. Quality of the alignment and Assignment rate to features (RNA-seq data)

Table S4. Results of the Differential Expression Analysis of the comparison 0 vs 1 mM EtOH at 6h

Tables S5A and S5B. Lists of the 386 functionally annotated DEGs for the 6h comparison, 1 mM versus 0mM: 254 Down-Regulated DEGs (A) and 132 Up-Regulated DEGs (B)

Table S6. Overall GO enrichment analysis results for the up-regulated DEG list (132 genes).

Table S7. Overall GO enrichment analysis results for the down-regulated DEG list (254 genes).

Table S8. Details of the up-regulated DEGs within the enriched GO term "Response to heat", resulting from the GO enrichment analysis.

Table S9. Details of the down-regulated DEGs within the enriched GO term "Secondary metabolism process", resulting from the GO enrichment analysis.

Supplementary Protocol

Supplementary protocol. ReliaPrep™ RNA Tissue Miniprep System protocol, optimised for RNA extractions on grapevine cell cultures.

Acknowledgments

We thank EUR TULIP and the Occitanie Region for the PhD grant to A. Diot. We also thank P. Thuleau for providing the *Arabidopsis thaliana* Col-0 seeds, L. Lemonnier and D. Saint-Martin for help in cell and plant cultures. T. Gillet for his help with oxygen measurements, and R. Althiab-Almasaud and P. Fraysse for their help with RNA-seq sample preparation. Then we want to thank M. Bouzayen and J. Pirrello for GBF team co-direction and side-funding.

Author contributions

C.C. and A.Diot conceptualised the project and designed the research. A.Diot performed the experimentations. G.M. and A.Djari performed the RNA-seq data cleaning, reads alignment and quality assessment of the reads. A.Diot, E.M. and G.M. performed the analyses on R and O.D.V., G.M. and A.Diot made the R graphs. A.Diot and C.C. wrote the original manuscript draft, and all co-authors contributed to editing the manuscript. A.Diot, S.B. and C.C. were involved in funding acquisition, C.C. and S.B. were involved in A.Diot's PhD supervision.

Conflict of interest

The authors declare they have no conflict of interest.

Funding

This study was supported by the École Universitaire de Recherche TULIP-GS (ANR-18-EURE-0019), which provided half of a PhD grant to A. Diot. All authors are also grateful to the Occitanie Region for funding the other half of the PhD grant to A. Diot. Additionally, we acknowledge the support from Université Paul Sabatier, CNRS, and Toulouse INP for their contributions to our research.

Data availability

The data underlying this article will be shared upon request to the corresponding author.

Special Issue

This article is part of the Special Issue 'Grapevine'. See the companion article Ait Kaci *et al*., 2024.

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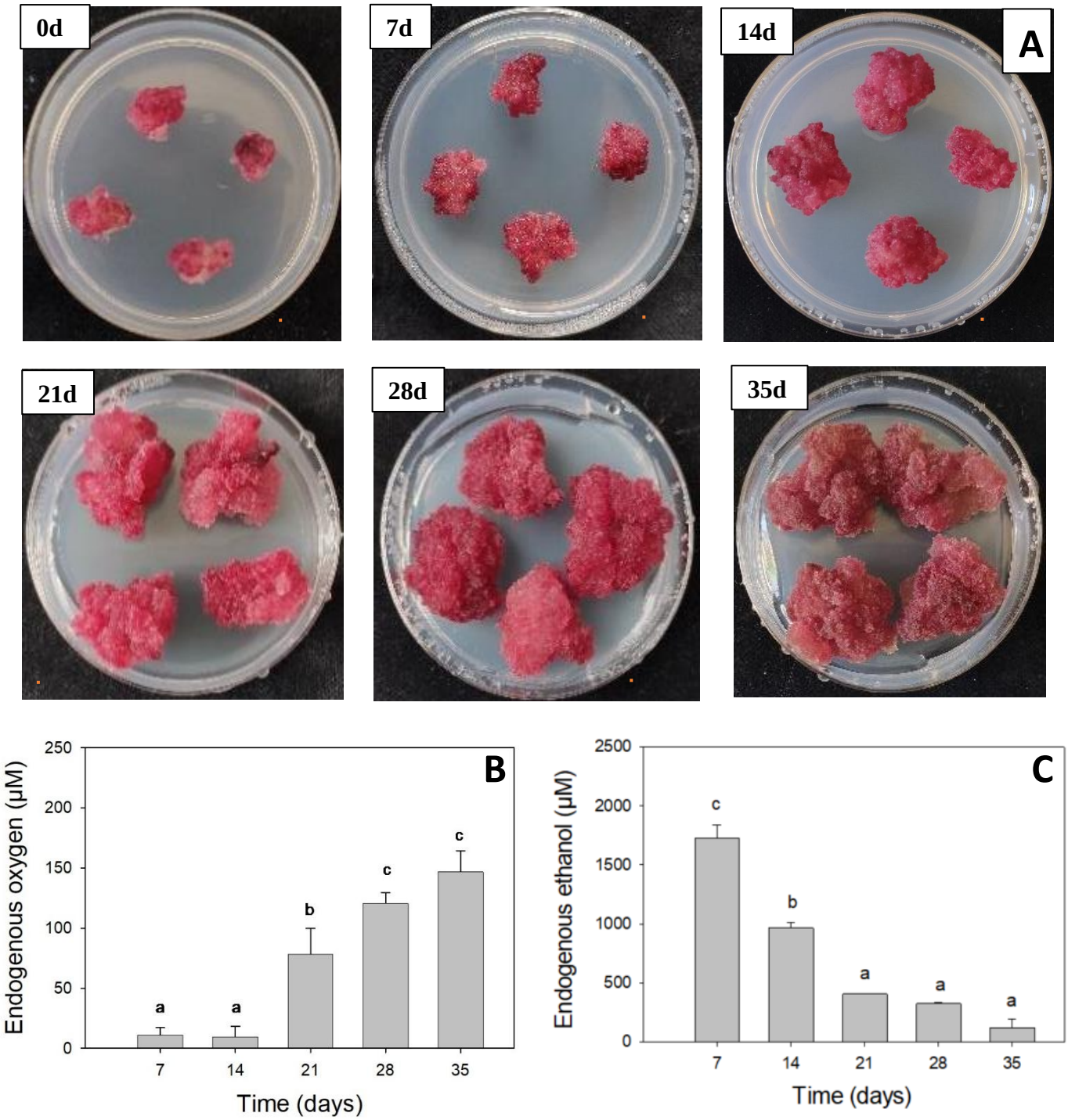

Fig. 1. A) Photographs of Gamay calli at 0 day, 7 days, 14 days, 21 days, 28 days, and 35 days after subculturing onto Petri dishes. The calluses selected are representative of the general development observed. Scale: orange bar represents 1 cm. **B)** Dissolved O_2 levels in callus at a distance of 2100 µm towards the callus centre as a function of development time (0, 7, 14, 21, 28 and 35 days), n = 4 biological replicates. **C)** Ethanol concentration as a function of development time (0, 7, 14, 21, 28 and 35 days), n = 3 biological replicates. Error bars represent standard error and different small letters indicate a significant difference at $p < 0.05$ by multiple comparisons using Fisher's LSD for endogenous oxygen content and Tukey's HSD for endogenous ethanol content.

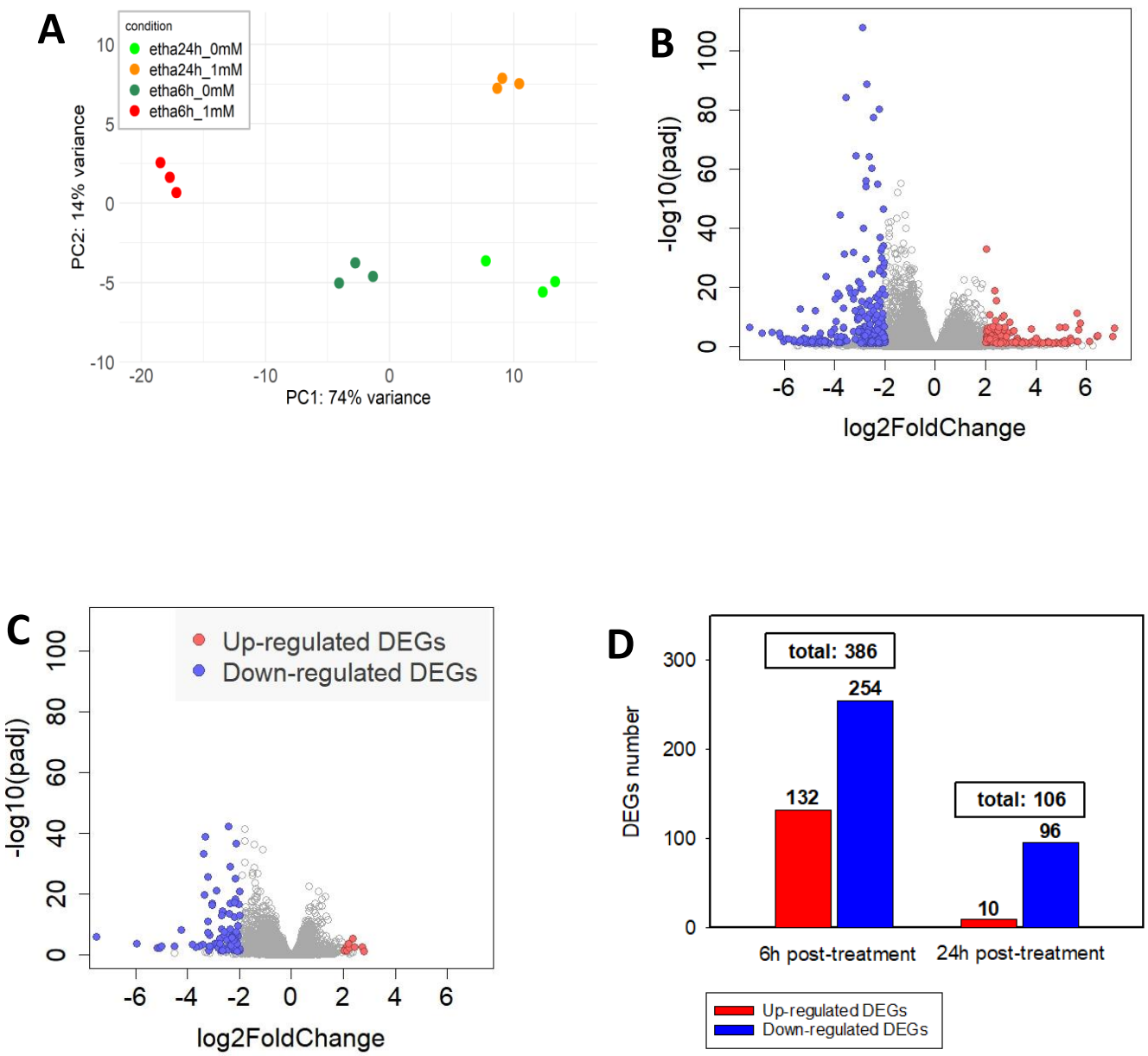

Fig. 2. **A)** Principal Analysis Component of transcriptomic profiles of Gamay Fréaux grape cell cultures 6 and 24 hours after a control 0 mM (green and dark green) or 1 mM (orange and red) ethanol treatment. Every symbol represents one replicate. **B)** Volcano plot of 24655 genes for differential gene expression, at 6 hours post-treatment. **C)** Volcano plot of 24655 genes for differential gene expression, at 24 hours post-treatment. For both Volcano plots: cell cultures treated with 1mM EtOH versus control cell cultures. Scattered points represent genes: the x-axis is the Log2FoldChange for the comparison of EtOH treated cells vs control cells, whereas the y-axis is the negative log10 of adjusted p-values (where P is the probability that a gene has statistical significance in its differential expression). Differentially Expressed Genes (DEGs) that are significantly upregulated (Log2FoldChange > 2 and adjusted p-value < 0.05) are marked in red, while significantly downregulated genes (Log2FoldChange < -2 and adjusted p-value < 0.05) are marked in blue. Genes that do not meet both the significance threshold and one of the log2FoldChange cutoffs are shown in grey. **D)** Comparative Analysis of DEG number for the comparison 1mM EtOH versus control, at two time points. The DEGs shown in the figure are any genes with a p-adj < 0.05 and a Log2FoldChange > 2 for the up-regulated DEGs or Log2FoldChange < -2 for the down-regulated DEGs.

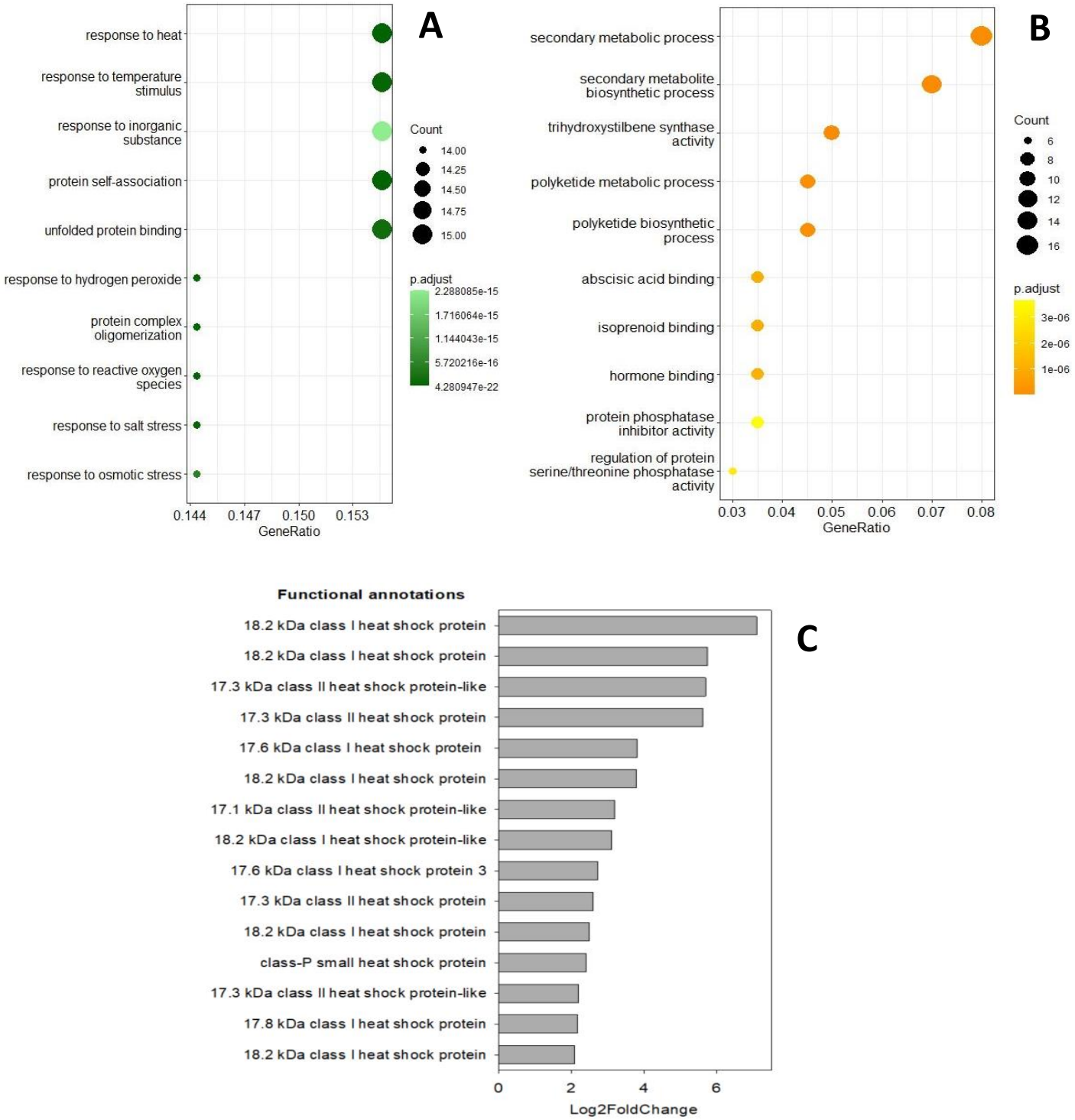

Fig. 3. Dot plots of the Gene Ontology (GO) enrichment analysis of **A)** up-regulated DEGs and **B)** down-regulated DEGs for the comparison of 1mM EtOH versus 0mM at 6 hours post-treatment. DEGs used for the GO enrichment analysis are genes with p-adj < 0.05 and cutoffs as follows: Up-regulated DEGs–with a Log2FoldChange > 2 and down-regulated DEGs with a Log2FoldChange < -2. The size of the dots represents the number of genes in the significant DE gene list associated with the GO term and the colour of the dots represent the enrichment P-adjusted value. **C)** Details of the 15 up-regulated DEGs within the enriched GO class of "Response to heat "and ordered by their respective Log2 Fold Changes for a 1 mM ethanol treatment compared to control, at 6 hours post-treatment.

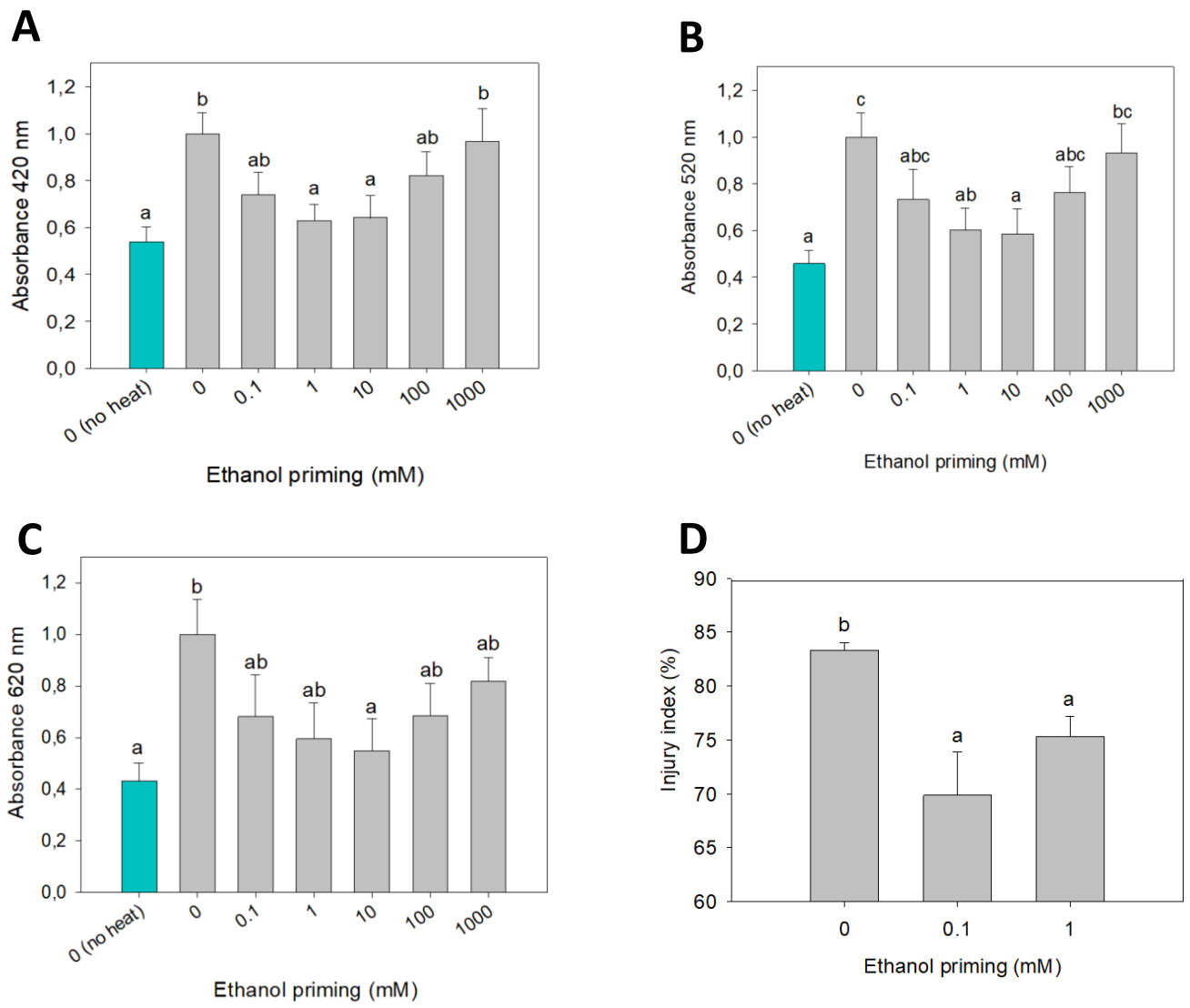

Fig. 4. Damages induced by heat stress in *Vitis vinifera* Gamay Fréaux cell cultures and *Arabidopsis thaliana* Col-0 seedlings, as a function of pretreatments with various ethanol concentrations. Pigments released from Gamay cell cultures, absorbing at **A)** 420 nm, yellow range, **B)** 520 nm, red range and **C)** 620 nm, blue range, following a 47°C heat-stress (see material and methods for details). Results are means ± SE, n = 8 biological replicates, small letters indicate a significant difference ($p < 0.05$) between groups, multiple comparisons by Fisher's LSD. **D)** Injury index of Arabidopsis seedlings based on ion leakage measurements following a 43°C heat-stress (see material and methods for details). Results means + SE, n = 7 biological replicates, small letters indicate a significant difference ($p < 0.05$) between groups, multiple comparisons by Tukey's HSD.